9 Pathophysiology of Corneal Graft Rejection

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General Issues

The most common form of solid organ transplantation in the United States is corneal allotransplantation [1]. Each year, approximately 40,000 patients in the United States receive a corneal transplant for vision rehabilitation [1]. The success rate in patients that are deemed "low risk" is extremely high, greater than 90% with the use of topical steroidal therapy alone. Much of the rationale for this high success rate is attributed to the immune privilege of both the cornea and anterior segment [2]. On the other hand, "high risk" patients (10–30% of hosts) with vascularization of their cornea preoperatively have a corneal graft failure rate between 60–90% [3–6]. This is particularly relevant in other countries where the percentage of "high risk" patients who receive a corneal transplant is closer to 90% [7, 8]. While systemic immunosuppression to minimize immunologic rejection in these high-risk patients has shown some efficacy in the literature, their usage has been limited by the complexity of the regimens as well by associated systemic complications associated with these therapies [5, 7, 8].

The most common cause of corneal graft rejection is immunological rejection [2, 3, 7, 9]. In order for immunological rejection of a corneal allograft to occur, the coordinated recruitment and infiltration of effector T cells that are alloantigen-primed are essential steps [10, 11]. When allo- specific T cells infiltrate into the corneal allograft, apoptosis and graft rejection result [12]. Therefore, the prevention of T cell-mediated immune rejection is the primary goal of postoperative medical therapy [2, 4].

Penetrating Keratoplasty vs. Endothelial Keratoplasty

Over the past decade, rates of endothelial keratoplasty have increased dramatically [13]. This surge is due, in part, to reports that endothelial keratoplasty procedures enjoy lower rates of rejection relative to penetrating keratoplasty [14–16]. However, in the high-risk setting (previous penetrating keratoplasty or corneal neovascularization), corneas are often scarred and thus require more extensive surgery than endothelial keratoplasty alone [17]. Furthermore, endothelial keratoplasties

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performed in high-risk hosts have substantially higher rejection rates than those performed in low-risk hosts compared to, and in some cases exceeding, those for penetrating keratoplasty [18, 19].

Risk Factors

Normally, the cornea and anterior segment enjoy immune privilege for numerous reasons [8]. Indeed, in a physiologically normal eye, the cornea is devoid of both lymphatics and blood vessels. Furthermore, there is a relative paucity of antigen presenting cells within the cornea. The continuous expression of Fas ligand (FasL) helps to promote apoptosis of immunocytes bearing Fas. Immunosuppressive cytokines circulating in the aqueous humor (such as TGFβ, α–MSH and VIP) promote immune quiescence [8]. However, when inflammation of the cornea or anterior segment occurs, this results in the breakdown of the vital blood-eye barrier which allows for violation the immune privilege found in normal corneas and anterior segments.

Blood Vessels

A severe inflammatory response can result in corneal vascularization, which permits immune rejection of the corneal allograft. This inflammatory response can result from a variety of etiologies including viral or bacterial infections, trauma, or rejection of a previous corneal graft [8]. Patients who have suffered from these inflammatory conditions are deemed to be at a higher risk of rejection and failure than patients who a receive a transplant for conditions such as keratoconus, where the patients often have a non-vascularized recipient corneal bed.

Vascularization of the corneal bed preoperatively is known to worsen the prognosis of a corneal transplant [6]. Studies have shown that the degree of corneal vascularization in the recipient cornea is directly proportional to the incidence of graft rejection and failure [3]. Additionally, in more highly vascularized corneas, the average time between the surgical transplantation and allograft rejection has been shown to be significantly shorter than in those recipients with less preoperative corneal vascularization [3].

Anterior Synechia

Preexisting anterior synechia is thought to be a poor prognostic sign in patients undergoing a keratoplasty. Studies suggest that fewer corneal allografts remained clear in patients with anterior synechia, and additionally that the risk of allograft rejection was higher [4, 6, 20].

Anterior Chamber Inflammation

Preoperative nonspecific anterior chamber inflammation, or persistent postoperative inflammation, is considered to predispose patients to allograft rejection [4].

Chemical Burns

In the Collaborative Corneal Transplantation Studies, eyes that had suffered prior chemical burn injuries had the highest rate of corneal allograft failure from all causes [6].

Loss of Immune Privilege

Other risk factors for graft failure are related to the loss of immune privilege of either the cornea or anterior chamber. The risk of allograft rejection or failure increases in patients who have a lack of aqueous suppressive factors, loss of expression of FasL, or loss of anterior chamber-associated immune deviation which normally would serve to prevent the suppression of delayed type hypersensitivity reactions to alloantigens.

ABO Blood Group Incompatibility

The rate of corneal rejection from any cause has been shown to be approximately 10% lower in

ABO-matched donors as compared to ABO-incompatible donors. Whereas HLA-antigen matching has not been shown to reduce the rates of graft failure, transplanting ABO-compatible corneas may be effective at decreasing the risk of allograft failure [5].

Prior Ocular Surgery

While prior anterior segment surgery is considered to increase the risk of rejection, the strongest correlation between a prior ocular surgery and allograft rejection is having undergone a prior corneal transplant. Furthermore, the incidence of allograft reaction increases and the time to onset of reaction decreases with each additional prior graft [3, 6].

Pathophysiology

Studies of transplant immunobiology have elucidated the complex sequence of molecular and cellular events that drive the immune rejection of corneal grafts. Initially, the tissue damage that occurs with surgery triggers an innate immune response, with infiltration of antigen-presenting cells into the cornea and the local release of pro-inflammatory factors [21]. Having acquired MHC class II and co-stimulatory molecules in this inflammatory milieu, APCs egress from the cornea and are trafficked via lymphatic vessels to draining lymph nodes [22]. Here, mature APCs present donor antigens to naïve host T cells, prompting the differentiation and clonal expansion of graft attacking type 1 T-helper (Th1) cells. The events leading to the generation of IFNγ-secreting $CD4^+$ Th1 cells in the lymph nodes can be understood as the "afferent arm" of the alloimmune response. Following a chemokine gradient, Th1 cells migrate via blood vessels toward the graft site, where they employ a variety of mechanisms to mount a delayed-type hypersensitivity immune response against the allogeneic corneal tissue (i.e., the "efferent" arm of the immune response) [23]. However, these pro-inflammatory pathways are modulated by an array of intrinsic immunoregulatory responses that promote graft tolerance, including regulatory T cells, tolerogenic dendritic cells, immunosuppressive epithelium-derived molecules, and neuropeptides [24]. It is the balance between these effector and regulatory immune mechanisms that determines whether allogeneic corneal grafts undergo immune rejection.

APC Activation

There are two pathways by which alloantigens are presented by APCs from the site of transplantation to T cells: the direct and indirect pathways. In the indirect pathway, recipient-derived APCs present antigens to T cells and thereby induce allosensitization [2]. This indirect pathway is considered to play a significant role in corneal allograft rejection although it is less efficient at doing so than the direct pathway [25, 26]. Donor-derived dendritic cells (DC) serve as the APCs in the direct pathway. This pathway is considered to play more of a role in high-risk patients and is approximately 100 times more effective than the indirect pathway. The increased effectiveness is a result of allogeneic MHC antigens bypassing the involvement of host APCs by directly activating naïve T cells [2, 27]. A single donor-derived DC is able to stimulate 3000 allospecific primed T cells [28, 29]. This priming is a pivotal step in rejection, and conditions that increase the number of donor-derived APCs, such as corneal neovascularization, will further increase the risk of corneal allograft rejection [2].

Effector Mechanisms

Chemokines are a superfamily of heparin-binding chemoattractant cytokines that direct leukocyte trafficking. One family of chemokines, CXC chemokines, recruits neutrophils and macrophages, along with other potent chemokines such as IP-10, Mig and I-TAC which all attract activated T cells. This family of CXC chemokines includes three chemokines, CXCL1/KC, CXCL9/Mig, and CXCL10/IP10 that are known to be produced

in high-risk allografts with corneal vascularization [30]. Additionally, these neutrophil attractant chemokines (i.e., CXCL1/KC) are upregulated in high- risk corneal allografts by pro-inflammatory cytokines like TNF-α and IL-1α, produced at the site of transplantation.

CXCL1/KC acts by recruiting neutrophils early and thereby causes acute damage to the allograft. The infiltrating neutrophils mark the corneal graft which then directs the recruitment of allo-specific effector T cells and other leukocytes. It also results in late production of CXCL9/Mig and CXCL10/IP10 and promotes the infiltration of T cells, macrophages, and natural killer (NK) cells into the allograft [30]. In fact, if CXCL1/KC is neutralized, graft survival increases, demonstrating that both the early infiltration of neutrophils and late recruitment of alloantigen-reactive T cells play an important role in allograft rejection [30].

Two of the IFN-γ-induced CXCR3 binding chemokines, CXCL9/Mig and CXCL10/IP10, are potent chemoattractants for allo-specific activated T cells bearing the CXCR3 chemokine receptor [30]. When CXCL9/Mig is neutralized, allograft survival has been shown to increase. CXCL10/IP10 is slightly more complicated and there is thought to be a regulatory component to rejection controlled by CXCL10/IP10, as complete neutralization of the chemokine has been shown to result in a faster and more robust rejection of the allograft.

Immune Regulation

Regulatory T Cells

Regulatory T cells (Tregs) are critical mediators of immune tolerance, and function by a variety of mechanisms to maintain immunological unresponsiveness to alloantigens [31]. These mechanisms include (i) the release of immunoregulatory cytokines, (ii) modulation of dendritic cell (DC) function, (iii) induction of cytolysis (via granzyme-B and perforin-dependent pathways), and (iv) competition for metabolic substrates such as IL-2.

The promotion of graft survival by of $CD4^+CD25^+Foxp3^+$Tregs has been demonstrated in adoptive transfer experiments using a mouse model of corneal transplantation [32–34]. Notably, expression of the transcription factor Foxp3 has been shown to be far more relevant to the immunoregulatory capacity of Tregs than frequency, with high levels of Foxp3 expression associated with increased immunosuppressive cytokine production and greater suppression of effector T cell activation [32]. Although Tregs are known to limit the immune response both at the site of inflammation and draining lymphoid tissue [35], evidence suggests that Tregs primarily modulate the immune response to corneal alloantigens by limiting T cell priming in the draining lymph nodes [32]. Following corneal transplantation, the Tregs of allograft acceptors, but not rejectors, have been shown to colocalize with APCs in the paracortical areas of draining lymph nodes [36]. Furthermore, Tregs from allograft acceptors express higher levels of the chemokine receptor CCR7 than allograft rejectors [36]. CCR7 is known to mediate the migration of Tregs to the paracortical areas of draining lymph nodes via high endothelial venules, where they suppress the priming of T cells by DCs [37]. Of note, the amplification of CCR7 expression by ex vivo culture of naïve Tregs with CCL21, followed by intravenous infusion of manipulated Tregs, has been shown to be an effective strategy to enhance Treg homing to the lymph nodes and promote corneal transplant survival [37].

The majority of $Foxp3^+$Tregs are generated in the thymus, and are referred to as thymus-derived Tregs (tTregs) [38]. This subset contrasts with Tregs generated from $Foxp3^-$ conventional T cells in peripheral tissues, termed peripherally derived Tregs (pTregs) [38]. Using a high-risk model of murine corneal transplantation in which intrastromal sutures were placed prior to transplantation to induce host bed inflammation and angiogenesis, it has been shown that pTregs (but not tTregs) are susceptible to dysfunction in the inflammatory microenvironment [34]. Indeed, pTregs sourced from high-risk hosts exhibited decreased suppression of conventional Tregs relative to low-risk hosts, and were also associated

with decreased expression of the immunoregulatory cytokines IL-10 and TGF-β but increased expression of the pro-inflammatory cytokine IFN-γ [34]. These data suggest that although pTregs promote tolerance to allografts in uninflamed host beds, they are in fact liable to *promote rejection* in the high-risk setting through the secretion of pro-inflammatory factors. This observation is consistent with evidence that Tregs exhibit considerable phenotypic plasticity, and may repolarize toward different fates according to cues from their microenvironment [39, 40].

Treg plasticity has been investigated in corneal transplantation using a double transgenic mouse model that permits Foxp3 lineage tracing [41]. This study demonstrated that a fraction of Tregs lost expression of Foxp3 in the inflamed setting, becoming IFNγ$^+$ or IL-17$^+$ "exFoxp3" cells that were phenotypically identical to effector Th1 or Th17 cells, respectively [41]. Moreover, the investigators showed that pTregs are particularly liable to conversion to exFoxp3 cells [41]. Tregs have developmental similarities with Th17 cells and reflected in their mutual requirement for TGF-β, and both cell types exhibit considerable plasticity [42]. These observations are highly pertinent to transplantation immunology, not least given reports describing a Th17-mediated alternative pathway of allograft rejection [43, 44]. Interestingly, studies of the effect of Th17 cells in murine corneal transplantation have inconsistent results, with some groups describing a disease-promoting role for Th17 cells [45], but others reporting exacerbated graft rejection following depletion of the Th17-associated cytokine IL-17A [46]. The plasticity and functional adaptability of Tregs have important implications for cell-based therapeutic strategies to increase allograft survival, in consideration of the risk that therapeutic Tregs may convert to pro-inflammatory phenotypes.

Tolerogenic Dendritic Cells

In addition to Treg-based therapies, there has been research interest in the use of ex vivo-manipulated DCs to promote transplant tolerance [47, 48]. In a murine model of corneal transplantation, CD200R3$^+$ regulatory DCs (DCregs, generated ex vivo by culturing bone marrow cells with GM-CSF, IL-10, and TGF-β) have been shown to promote corneal allograft survival [49]. Indeed, DCreg administration was observed to reduce CD4$^+$IFN-γ$^+$ T cell frequencies and increase Foxp3 expression by Tregs [49]. In a separate murine corneal transplantation study that defined tolerogenic APCs (tolAPCs) as CD11c$^+$MHC class II^{lo}CD40^{lo}CD86^{lo} cells, subconjunctival injection of recombinant IL-10 and TGF-β into donor eyes was observed to augment the population of corneal tolAPCs [50]. Furthermore, transplantation of these tolAPC-enriched corneas decreased CD4$^+$IFN-γ$^+$ T cell frequencies, diminished graft infiltration of CD45$^+$ and CD4$^+$ cells, and significantly promoted corneal graft survival [50]. Whether by the administration of ex vivo-manipulated cells or by cytokine conditioning of donor corneal tissue, these reports offer strategies to augment tolerogenic APC populations and thus promote graft survival.

Corneal Avascularity

The healthy human cornea does not contain blood and lymphatic vessels. The absence of lymphatics limits the trafficking of APCs to the draining lymph nodes (i.e., the afferent arm of the immune response), and the absence of blood vessels curbs the trafficking of effector T cells to the graft site (i.e., efferent arm) [24]. Still, the cornea can become vascularized due to a range of corneal pathologies and surgical trauma. There is an array of antiangiogenic and antilymphangiogenic factors present at the ocular surface that maintain corneal avascularity, thereby impeding allosensitization and promoting graft tolerance. The corneal epithelium secretes an endogenous spliced variant of soluble VEGFR-2 (sVEGFR-2) that suppresses lymphangiogenesis by blocking VEGF-C, thereby limiting the afferent arc of the immune response [51]. As illustration of its tolerogenic properties, intracorneal administration of sVEGFR-2 has

been shown to increase graft survival by more than double in a murine model of corneal transplantation [51]. Inflammatory angiogenesis is suppressed by sVEGFR-1 and VEGFR-3 that are constitutively expressed by the corneal epithelium, thus curbing the efferent arc of the immune response [52, 53]. Endostatin is a 20-kDa collagen fragment expressed by multiple ocular tissues, including corneal and conjunctival epithelial cells [54]. By inhibiting neovascularization and delaying the recruitment of allospecific T cells, endostatin has been shown to prolong corneal allograft survival in murine studies [54]. The corneal epithelium expresses the immunoregulatory molecules thrombospondin (TSP)-1, programmed death ligand-1 (PD-L1), and pigment epithelium-derived factor [PEDF], which also suppress corneal angiogenesis [55–57] and lymphangiogenesis [58]. By inhibiting the development of lymphatic and blood vessel networks, this amalgam of factors work in concert at the cornea to restrict the alloimmune response.

Corneal Epithelial Immunoregulatory Factors

In addition to limiting hemangiogenesis and lymphangiogenesis, immunoregulatory factors expressed by the corneal epithelium have been shown to modulate the alloimmune response in a variety of ways. Corneal epithelial cells express Fas ligand (FasL), which engages the cell death surface receptor Fas, thus mediating apoptotic cell death via caspase activation [59]. In murine corneal transplantation studies using $FasL^-$ mice, $FasL^-$ corneas have been observed to have numerous inflammatory cells without apoptosis, associated with graft rejection rates of almost 100% [60, 61]. In contrast, $FasL^+$ grafts were observed to contain apoptotic mononuclear cells with an acceptance rate of approximately 50% [60, 61]. These studies provide evidence for the importance of FasL expression in regulating the alloimmune response. PDL-1 is constitutively expressed at high levels by the corneal epithelium, and through its interaction with PD-1 on T cells has been shown to suppress T cell proliferation, limit IFN-γ production and promote corneal allograft survival [62, 63]. A role has been reported for interleukin-1R antagonist (IL-1Ra), also expressed constitutively by corneal epithelium, in maintaining ocular immune quiescence by suppressing APC migration [64]. Topical application of exogenous IL-1Ra has been shown to promote graft survival in both the normal-risk and high-risk settings [65]. TSP-1 has been demonstrated to activate the immunoregulatory cytokine TGF-β [66]. TSP-1 is expressed by corneal epithelium as well as APCs, and has been shown to suppress allosensitization and promote graft survival in a murine model of corneal transplantation [67]. In this study, almost all TSP-1 null allografts were rejected, compared to 50% of wild-type allografts. TSP-1 null APCs expressed increased levels of MHC class II and CD80 maturation markers, indicating increased APC maturation in the absence of TSP-1 [67]. By the expression of these immunoregulatory molecules, the corneal epithelium helps to foster a tolerant immune microenvironment and promote allograft survival.

Neuropeptides

Recent research has shed light on the cross talk between the immune and nervous systems in the setting of corneal transplantation. The neuropeptides alpha–melanocyte stimulating hormone (α–MSH) and vasoactive intestinal peptide (VIP), constitutively expressed in the cornea, have been shown to promote the survival of allogeneic grafts in murine studies [68, 69]. Subconjunctival injection of α–MSH following corneal transplantation has been reported to decrease graft infiltration of mononuclear and polymorphonuclear cells, and to significantly reduce graft rejection [68]. VIP has been shown to suppress IFN-γ– and TNF-α–induced corneal endothelial cell loss, preserve corneal endothelial cell density and promote allograft survival in high-risk murine model of corneal transplantation [69]. The immunoregulatory roles of other neuropeptides (including calcitonin gene-related

peptide and neuropeptide Y) at the cornea have been described and are the subject of ongoing investigations [70].

Future Prospects in Regulating Immunity and Cytoprotection

Despite substantial advances in our understanding of the effector and regulatory mechanisms that underlie the alloimmune response to corneal grafts, there is a deficit of novel therapeutics available to the corneal transplant surgeon. Indeed, corticosteroids remain the first-line therapy in most instances, despite their considerable toxicity and limited efficacy in high-risk hosts (with previous rejection episodes or vascularized graft beds) [71]. However, there are a number of promising avenues for the translation of bench-side discoveries to the clinic.

Cell-based therapies include the local or systemic administration of immunomodulatory cells such as Tregs [32, 36, 72, 73] and mesenchymal stem cells (MSCs) [74, 75]. However, the translational potential of cell-based therapies is constrained by multiple issues, including (i) the challenge of consistent production of clinical-grade cells, (ii) strict regulatory standards, (iii) substantial cost (both resources and time) of complying with regulatory standards, and (as discussed previously) (iv) phenotypic and functional plasticity of immune cells. Various approaches to circumvent these issues have been proposed, including the in vivo expansion of immunoregulatory cells such as Tregs [33, 76], and the identification of critical anti-inflammatory molecules secreted by immunoregulatory cells that are of potential therapeutic value (e.g., MSC secretion of tumor necrosis factor-α stimulated gene/protein 6 [75]). Alternative strategies to enhance the survival of corneal allografts include targeting heme/lymphangiogenesis with anti-VEGF agents [77–79]. Neuropeptides are also candidate therapies; for example, local VIP administration following high-risk corneal transplantation has been shown to decrease opacity and promote graft survival with increased corneal endothelial cell density observed [69]. These approaches may permit the development of novel treatments that decrease rates of corneal transplant rejection and/or graft endothelial cell loss, particularly in the high-risk host.

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